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14. ABSTRACT

SRC-3/AIB1 is a steroid receptor coactivator with potent growth promoting activity. It is overexpressed in a number of cancers and has been shown to have oncogenic potential. An important mechanism for the regulation of SRC-3 coactivator 'activity' is its site-specific phosphorylation status, which is controlled by extracellular signals that stimulate specific protein kinase signaling pathways. It is well known that protein kinases such as aPKC are frequently overexpressed in cancers and that phosphorylation of SRC-3 can contribute to its oncogenic potential. However, it is unclear whether protein kinases can directly regulate SRC-3/AIB1 cellular protein levels. In the present study, we showed that atypical protein kinase C (aPKC) phosphorylates and stabilizes SRC-3; increased intracellular levels of the coactivator result in significant enhancement of steroid receptor transcription activity. The aPKC stabilization effect requires the presence of estrogen receptor. Using a variety of estrogen receptor mutants we found that this stabilization occurs primarily in the nucleus and is dependent on a direct interaction between SRC-3 and estrogen receptor. Furthermore, we showed that aPKC-phosphorylated SRC-3 becomes resistant to degradation by the 26s proteasome in a cell-free degradation assay using all purified components of SRC-3, ER, and 26s proteasome. Taken together, our results suggest that, in addition to its role in activation of this important SRC-3 growth coactivator, phosphorylation also plays a role in regulating SRC-3 stability and turnover. Thus, aberrant levels of select protein kinases such as aPKC in cancer cells might contribute to the high levels of SRC-3 seen in endocrine cancers.

15. SUBJECT TERMS
seroid receptor coactivator, transcription, phosphorylation, signal transduction

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Table of Contents

INTRODUCTION:

Steroid receptor coactivator-3 (SRC-3/AIB1) is a member of the p160 coactivator family. It activates nuclear receptor mediated transcription through recruiting proteins such as CBP/p300 and CARM1 that have capability to modify histone. SRC-3 is overexpressed and amplified in a number of cancers including breast cancer (1). Recently, accumulated evidence also indicates an important role of SRC-3 in cancer development. Transgenic mice with SRC-3 overexpression were found to have extremely high tumor incidence (17). In contrast, SRC-3 knockout mice have significantly lower incidence of ras-induced mammary gland tumorgenesis (6). It appears that the level of SRC-3 protein is critical for tumorgenesis. A number of extracellular signals affect SRC-3 coactivator activity through activating downstream protein kinases, which eventually leads to SRC-3 phosphorylation. This post-translational modification was reported to contribute the oncogenic potential of SRC-3 (20). Protein kinases are often overexpressed in breast cancers and some kinases also serve as prognostic factors for breast cancer (7, 9, 11, 16). Protein stability is an important factor regulating protein function. Phosphorylation is often a way to regulate protein stability. Recently, it was shown that phosphoryylation of SRC-3 by p38MAP kinase is associated with its degradation in a RAR-dependent manner (5). However, it is still not clear whether kinase mediated SRC-3 phosphorylation could also modulate its protein stabilization since many kinases and SRC-3 are overexpressed in breast cancer. Here we demonstrate that atypical protein kinase C phosphorylates SRC-3 and this phosphorylation changes its conformation into a form that is more resistant to proteasome degradation, which eventually leads to protein stabilization.

BODY:

Atypical PKC stabilizes SRC-3 protein

To determine whether protein kinases have any effect on SRC-3 protein level, we co-transfected flag-SRC-3 with PKCζ, PKA catalytic subunit, IKKα or IKKβ into HeLa cells. Flag-ER was also co-transfected as a control. Shown in Fig.1A, PKCζ significantly increased SRC-3 protein level while other kinases have minor effects. PKCζ belongs to atypical protein kinase C subfamily of kinases. Another member of atypical PKC is PKCι. There are highly homologous with overall 72% amino acid identity (12). Unlike conventional and novel PKCs, atypical PKC is insensitive to phorbol ester, DAG or calcium stimulation (12, 13, 19). Atypical PKCs were found overexpressed in ovarian, prostate, lung cancers and liver, urine bladder carcinomas (3, 4, 14, 15, 18). We found that the effect of PKCζ on SRC-3 protein level requires its kinase activity since the kinase dead mutant of PKCζ does not have any effect on SRC-3 (Fig.1B). Similar results were also obtained with another atypical PKC, PKCι. RT-PCR results (Fig.1C) showed that transfected flag-SRC-3 mRNA level did not

increase when PKCζ was co-transfected, suggesting that the increased SRC-3 protein level is caused by change of SRC-3 protein stability instead of mRNA transcription. To examine whether PKCζ also affects endogenous SRC-3 protein level, ER positive breast cancer cells, MCF7 cell, were treated with siRNA against PKCζ. As shown in Fig.1D, PKCζ protein level was significantly reduced by the siRNA. SRC-3 protein level also decreased with reduced PKCζ level. Real time RT-PCR was also carried out to measure SRC-3 mRNA level when PKCζ was knocked-down. Similar to the results shown in Fig.1C, the endogenous SRC-3 mRNA level was not significantly altered by siRNA against PKCζ (Fig.1E). Since PKCζ does not affect SRC-3 transcription, we then examine whether PKCζ changes proteasome mediated SRC-3 degradation. As shown in Fig.1F, the proteasome inhibitor MG132 significantly increased flag-SRC-3 protein level and it abolished the effect of PKCζ on SRC-3. All these results suggest that atypical PKC stabilizes SRC-3 protein by preventing the proteasome mediated protein degradation.

The stabilization effect of PKCζ on SRC-3 requires its interaction with ER

The experiments shown in Fig.1 were always done in the presence of ER, either with transfected or endogenous ER. We also tested the effect of PKCζ on SRC-3 in the absence of ER or in the presence of progesterone receptor. Surprisingly, we found that PKCζ failed to stabilize SRC-3 in both cases (data not shown). It appears that ER is required for the stabilization effect of PKCζ on SRC-3. We then tested whether estrogen has any effect on this stabilization (Fig.2A). In the absence of estrogen, PKCζ wild type and its kinase dead mutant can not stabilize SRC-3 while the constitutive active mutant increased SRC-3 protein level. In the presence of estrogen, PKCζ wild type stabilizes SRC-3 while the effect of its constitutive active mutant was further augmented. Interestingly, ER protein level was also increased by the wild type and active mutant of PKCζ in the presence of estrogen. These results suggest that estrogen may have dual roles in the stabilization effect. First, it may activate PKCζ activity since only active PKCζ is able to stabilize SRC-3 in the absence of estrogen. Second, estrogen enhances the effect of PKCζ on SRC-3.

It was reported that PKCζ interacts with ER (2). Since ER is required in this stabilization, we then examined the interaction between PKCζ and SRC-3 or ER in the co-immunoprecipitation experiment. As shown in Fig. 2B, Both SRC-3 and ER interact with PKCζ and the interaction between SRC-3 and PKCζ does not require ER. We then tested the effects of different ER mutants in the SRC-3 stabilization. ER wild type, the mutant with AF1 domain deletion (ERΔAF1), the mutant with mutations in AF2 function (ERAF2m) or the mutant localized in the cytoplasm (ER(cyto)m) was co-transfected with flag-SRC-3 in the absence and presence of PKCζ. As shown in Fig.2C, ERΔAF1 mutant significantly reduced the stabilization effect while ERAF2m and ER(cyto)m abolished the stabilization. To determine whether the inability of ERAF2m and ER(cyto)m to support the PKCζ stabilization effect is due to their defect in the interaction with PKCζ or SRC-3, we did co-immunoprecipitation experiment (Fig.2D). V5- PKCζ and different ER mutants were co-transfected into

HeLa cells. The cell lysates were immunoprecipitated by anti-ER antibody and immunoblotted with anti-V5 antibody for detection of bound PKCζ. The results show that ERAF2m was still able to interact with PKCζ while ER(cyto)m failed to interact. Since ERAF2m can not interact with SRC-3, these results suggest that the interaction between ER and SRC-3 is important for the PKCζ stabilization effect and this stabilization occurs in the nucleus. To further confirm the importance of the ER-SRC-3 interaction. We also examined the effect of PKCζ on SRC-3 AAA mutant which contains mutations in three LXXLL motifs that are important for the interaction with nuclear receptors. The stabilization effect is significantly reduced (Fig.2E).

Since SRC-3 was shown to be a phosphor protein and PKCζ is a protein kinase, we then wanted to examine whether PKCζ can phosphorylate SRC-3 and whether ER has any function in this phosphorylation. In vitro PKCζ kinase assay was carried out using purified SRC-3 as a substrate. As shown in Fig.2F, PKCζ is able to phosphorylate SRC-3. The presence of ER increased and estrogen further enhanced the phosphorylation, suggesting that the interaction between ER, SRC-3 and PKCζ may put SRC-3 in an optimal conformation and facilitate the phosphorylation by PKCζ.

PKCζ protects SRC-3 from proteasome mediated degradation

SRC-3 can be degraded through both ubiquitin-dependent and independent pathways (8, 10). Since PKCζ directly phosphorylates SRC-3, we reason that this phosphorylation may change the conformation of SRC-3 into a form that is more resistant to proteasome degradation. To test this hypothesis we co-transfected flag-SRC-3 with ER, PKCζ or empty vectors into HeLa cells. Flag-SRC-3 purified from cell lysates by immunoprecipitation was then subjected to in vitro 26S proteasome degradation. As shown in Fig.3A, 26S proteasome efficiently degraded SRC-3 when either ER or PKCζ was present. When both ER and PKCζ were present, less SRC-3 was degraded. This protection effect requires PKCζ kinase activity since the presence of kinase dead mutant of PKCζ does not have this effect (Fig.3B). These results are consistent to the effect of PKCζ and ER on SRC-3 protein level in cells. It was shown recently that SRC-3 can also be degraded through REGγ mediated proteasome pathway (8). We then wanted to examine whether this PKCζ induced conformation of SRC-3 is also resistant to REGγ mediated degradation. Flag-SRC-3 purified from cell lysates was subjected to REGγ and 20S proteasome mediated degradation (Fig.3C). Similar to the results shown in Fig.3A, PKCζ also protected SRC-3 from degradation in this case. Although we showed that PKCζ can directly phosphorylates SRC-3, it does not necessary mean that the protection effect we observed is due to the direct phosphorylation of SRC-3 by PKCζ. To test this, purified baculovirus expressed SRC-3 was phosphorylated by PKCζ in vitro and the protein was then subjected to REGγ mediated degradation. In this assay PKCζ is the only kinase. As shown in Fig.3D, PKCζ still protected SRC-3 from degradation in this vitro purified system. These results suggest that PKCζ phosphorylates SRC-3 and induces its conformational change which is more resistant to degradation mediated by both ubiquitin dependent and independent proteasome pathways.

PKCζ increases nuclear SRC-3 protein level and enhances its coactivator activity

As shown above the cytoplasm localized ER mutant failed to support the stabilization effect of PKCζ on SRC-3, suggesting that this stabilization takes place in the nucleus. To confirm that nuclear SRC-3 protein really increased, we separated HeLa cell lysates into cytoplamic and nuclear fractions. The successful separation of cytoplasmic and nuclear compartments was shown by detecting nuclear protein PARP and the protein β-tubulin, most of which are localized in cytoplasm (Fig.4A). Under normal condition SRC-3 was localized in HeLa nucleus. When PKCζ was overexpressed in cells, nuclear SRC-3 protein level was dramatically increased while small amount of SRC-3 was also found in the cytoplasm. This may be due to the nucleus-cytoplasm shuttling of SRC-3. To confirm these results immunostaining of V5- PKCζ and HA-SRC-3 in HeLa cells was performed (Fig.4B). PKCζ is localized in both cytoplasm and nucleus. Consistent with the results shown in Fig.4A, SRC-3 was found to have increased protein level in the nucleus and it also showed cytoplasmic staining.

We then wanted to ask whether these increased SRC-3 protein in the nucleus are functionally active or not. To answer this question the transcription of endogenous ER target gene, pS2, was measured when cells were co-transfected with SRC-3 and PKCζ. The addition of PKCζ did not increase ER activated pS2 transcription without the co-transfection of SRC-3. When SRC-3 was transfected, PKCζ wild type significantly enhanced pS2 transcription in the presence of estrogen and the constitutively active mutant of PKCζ further augmented the transcription while the kinase dead mutant did not have any effect. These results suggest that the stabilized SRC-3 protein by PKCζ is transcriptionally active. PKCζ appears to boost SRC-3 activity by preventing its degradation.

In summary, we found that PKCζ phosphorylates and stabilizes SRC-3 protein. This effect requires the interaction between ER and SRC-3. ER increases the phosphorylation level of SRC-3 and the phosphorylation appears to change the conformation of SRC-3 into a form that is less degradable by proteasome. Furthermore, we showed that this stabilization occurs in the nucleus and PKCζ enhances ER mediated transcriptional activation by increasing the coactivator SRC-3 protein level.

KEY RESEARCH ACCOMPLISHMENTS:

Identify the kinase that leads to SRC-3 protein stabilization.

Determine the interaction between ER and SRC-3 is an important factor for the stabilization effect of atypical PKC.

Determine that the mechanism of atypical PKC stabilizing SRC-3 is to changing its conformation which is more resistant to proteasome mediated degradation.

Determine that the stabilization occurs in cell nucleus and the functional effect of this stabilization is to enhance SRC-3 coactivator activity.

REPORTABLE OUTCOMES:

Publications and Meeting Abstracts:

Yi, P., Wu, R. C., Sandquist, J., Wong, J., Tsai, S. Y., Tsai, M-J, Means, A. R., O'Malley, B.W. Peptidyl-prolyl isomerase 1 (Pin1) serves as a coactivator of steroid receptor by regulating the activity of phosphorylated steroid receptor coactivator 3 (SRC-3/AIB1). Mol. Cell. Biol. (2005) 25, 9687-9699.

Yi, P., Wong, J., Tsai, S.Y., Tsai, M-J, Sandquist, J., Means, A.R., and O'Malley B.W. Peptidyl-prolyl Isomerase 1 (Pin1) Serves as a Coactivator of Steroid Receptor-mediated Transcription by Regulating the Function of Phosphorylated Steroid Receptor Coactivator-3 The Program and Abstracts of the 87th Annual Meeting of the Endocrine Society (2005) p.616: P3-299.

Yi, P., Wong, J., Tsai, S.Y., Tsai, M-J, Sandquist, J., Means, A.R., and O'Malley B.W. Peptidyl-prolyl Isomerase 1 (Pin1) Serves as a Coactivator of Steroid Receptor-mediated Transcription by Regulating the Function of Phosphorylated Steroid Receptor Coactivator-3 Era of Hope Department of Defense Breast Cancer Research Program Meeting (2005) P35-21.

CONCLUSION:

We found that PKCζ phosphorylates and stabilizes SRC-3 protein. This effect requires the interaction between ER and SRC-3. ER increases the phosphorylation level of SRC-3 and the phosphorylation appears to change the conformation of SRC-3 into a form that is less degradable by proteasome. Furthermore, we showed that this stabilization occurs in the nucleus and PKCζ enhances ER mediated transcriptional activation by increasing the coactivator SRC-3 protein level. These findings provide one of the mechanisms that SRC-3 is overexpressed in cancers. They suggest that aberrant expression or abnormal activity of atypical PKC in cancers could result in highly concentrated and highly active SRC-3 protein in cells, which in turn activate a number of transcription factors and downstream target genes and eventually lead to tumor development. Our findings also provide a potential therapeutic target, atypical PKC, for the control of breast cancer development.

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APPENDICES:

Figure legends:

Fig.1 Atypical PKC stabilizes SRC-3 protein. (A) Flag-SRC-3 and ER expression vecotors were co-transfected with PKCζ, PKA, IKKα or IKKβ expression vectors into HeLa cells. The expression of flag-SRC-3 or ER was detected by western blot using anti-flag or anti-ER antibody. β-actin expression was included as a control. (B) Western blot analysis of flag-SRC-3 protein level when cells were co-transfected with constitutively active mutant of PKCζ or PKCι, or their kinase dead mutants. (C) RNA was extracted from cells transfected with flag-SRC-3 with or without co-transfection of PKCζ. RT-PCR was performed using flag-specific primer and SRC-3-specific primer to detect the mRNA level of transfected flag-SRC-3. A RT-PCR experiment without the addition of reverse transcriptase (RT) was included as a control. (D) Western blot analysis of endogenous SRC-3, ER and PKCζ protein level in MCF-7 cells treated with siRNA against PKCζ and scramble control siRNA. (E) real time RT-PCR was carried out to detect the mRNA level of endogenous SRC-3 in MCF-7 with or without PKCζ knocking-down. (F) PKCζ stabilizes SRC-3 protein in proteasome-mediated degradation pathway. HeLa cells transfected with flag-SRC-3

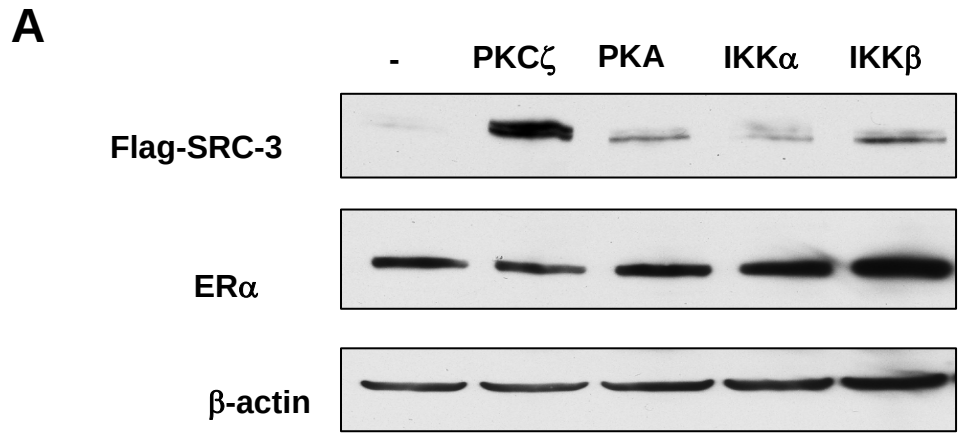
and PKCζ or its kinase dead mutant (DM) were treated with proteasome inhibitor MG132 or vehicle DMSO.

Fig.2 The stabilization effect of PKCζ on SRC-3 requires its interaction with ER. (A) Estrogen increases the PKCζ mediated stabilization effect. WT: wild type PKCζ, CAT: constitutively active mutant, DM: kinase dead mutant. (B) Co-immunoprecipitation experiment. Cells were transfected with V5- PKCζ and flag-SRC-3 or flag-ER. Cell lysates were immunoprecipitated by anti-flag antibody or normal IgG and western blot analysis was performed using anti-V5 antiobody. (C) The effects of different ER mutants on the PKCζ mediated stabilization effect. (D) Co-immunoprecipitation experiment detecting the interaction between PKCζ and different ER mutants. (E) PKCζ has significantly reduced stabilization effect on SRC-3 mutant which has mutations in three LXXLL motifs. (F) Autoradiograph of in vitro PKCζ kinase assay detecting phosphorylation of SRC-3 in the absence and presence of ER and estrogen.

Fig.3 PKCζ protects SRC-3 from proteasome mediated degradation. (A) in vitro 26S proteasome degradation assay. HeLa cells were transfected with flag-SRC-3 with or without co-transfection of either ER or PKCζ. Flag-SRC-3 was purified by immunoprecipitated from cell lysates and eluted by flag peptides. The purified flag-SRC-3 was then subjected to 26S proteasome degradation. (B) PKCζ kinase activity is required for its protection effect on SRC-3. The procedure was the same as described in panel A. (C) PKCζ protects SRC-3 from REGγ-mediated proteasome degradation. Similar procedure as described in panel A except that purified REGγ and 20S proteasome were used to degrade SRC-3. (D) PKCζ protects SRC-3 from REGγ-mediated proteasome degradation in in vitro purified system. Purified baculovirus expressed recombinant SRC-3 was phosphorylated by PKCζ in vitro. Purified REGγ and 20S proteasome were then added into the reaction and were incubated for indicated time period.

Fig.4 PKCζ increases nuclear SRC-3 protein level and enhances its coactivator activity. (A) Majority of increased SRC-3 protein by PKCζ are localized in the nucleus. HeLa cells transfected with flag-SRC-3 and PKCζ constitutively active mutant, its kinase dead mutant or empty vector were separated into cytoplasmic and nuclear fractions. Nuclear protein PARP and the protein β-tubulin whose majority is in cytoplasm were included in the western blot as controls. (B) Immunostaining of HA-SRC-3 in the absence or presence of exogenous expressed constitutively active PKCζ and its kinase dead mutant. (C) PKCζ enhances SRC-3 activated ER targeted gene transcription. HeLa cells were transfected with ER, SRC-3, PKCζ or its mutants in the absence or presence of estrogen. The transcription of pS2 gene was measured by real time RT-PCR on pS2 mRNA level.

Fig. 1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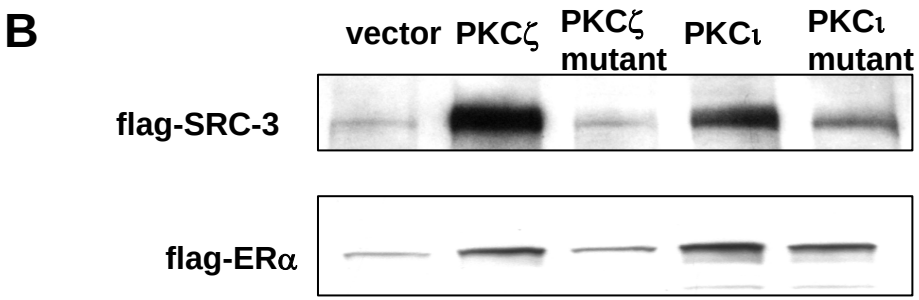
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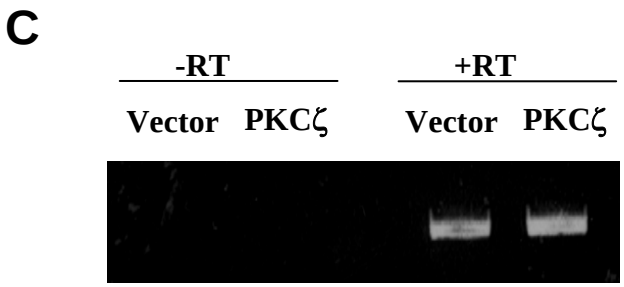
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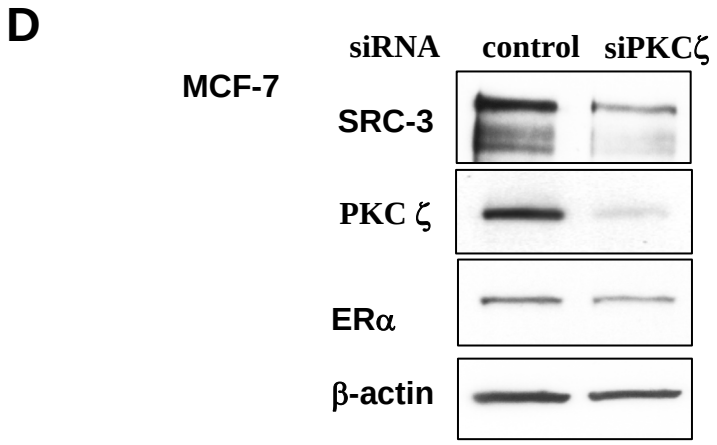

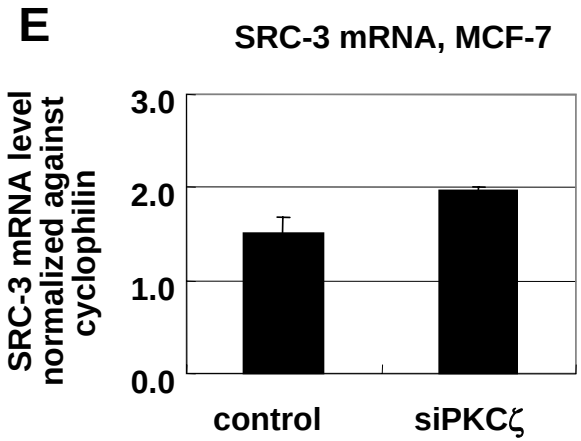

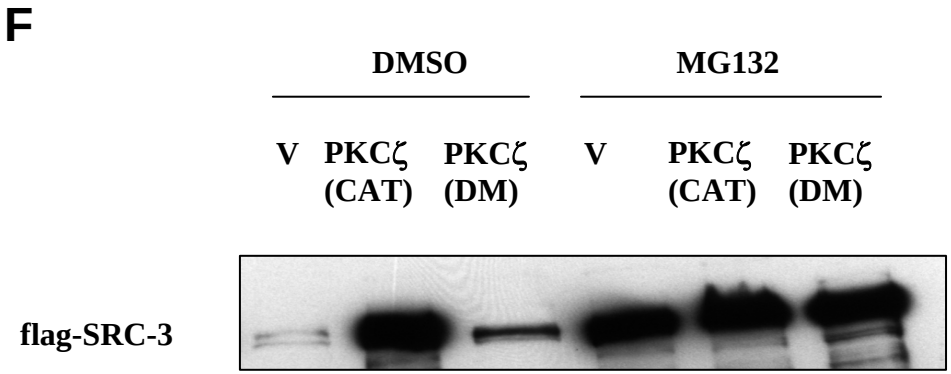

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Fig. 2

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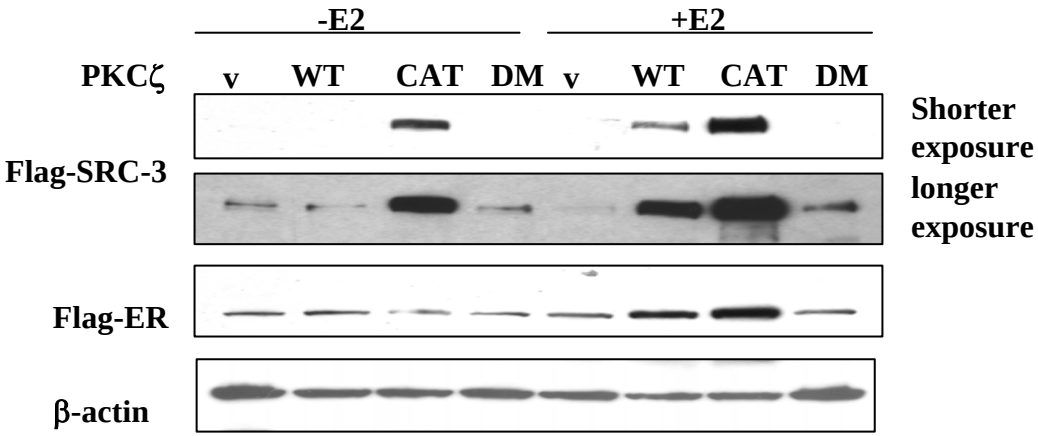
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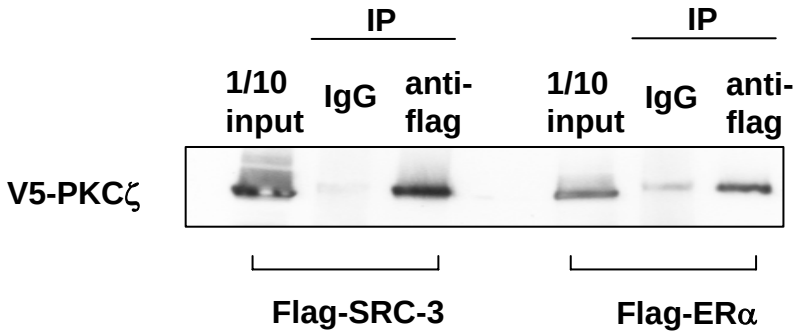
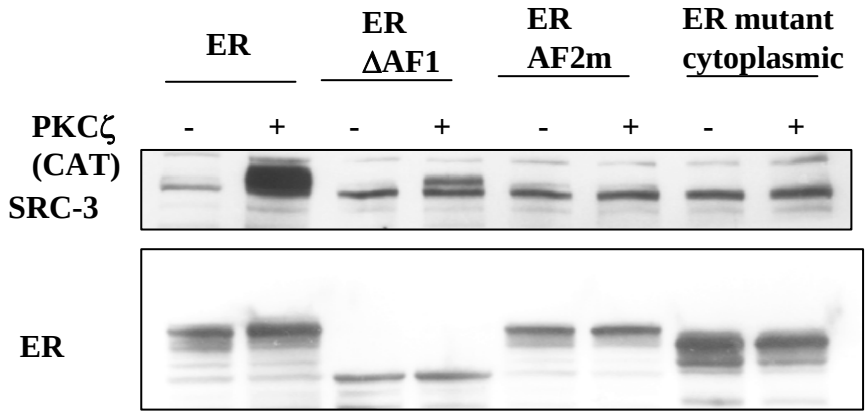
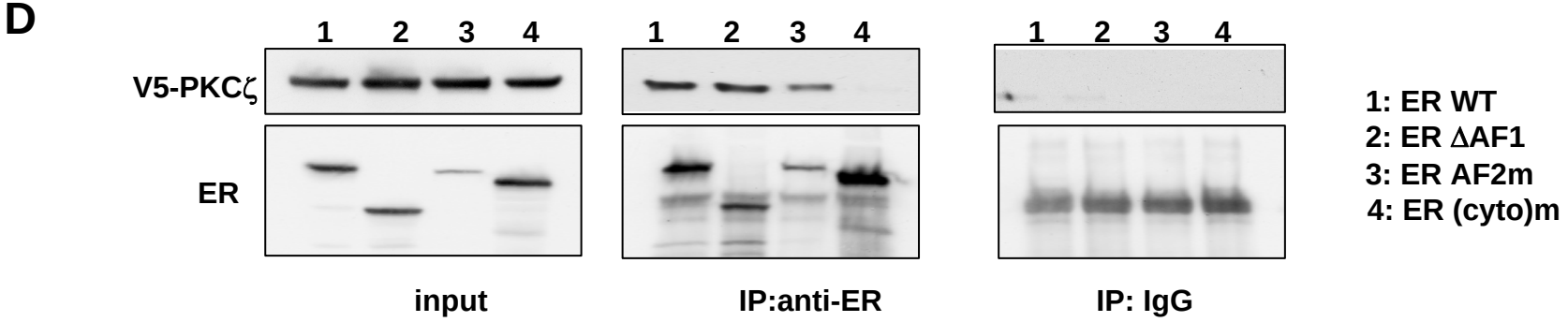
β-actin

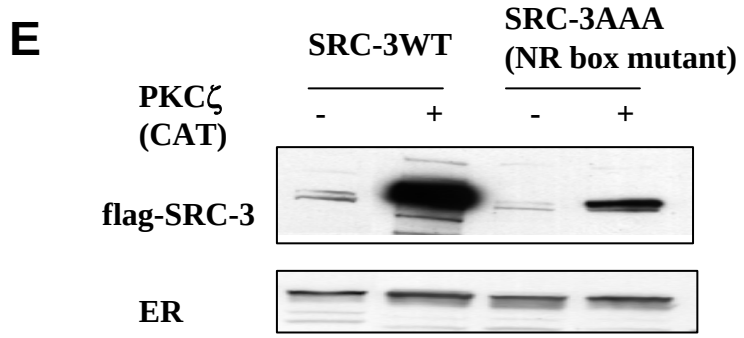
B


IP
IP
1/10 input
IgG
anti-flag
1/10 input
IgG
anti-flag
V5-PKCζ
Flag-SRC-3
Flag-ERα

C


ER
ER ΔAF1
ER AF2m
ER mutant cytoplasmic
PKCζ (CAT)
- + - + - + - +
SRC-3
ER





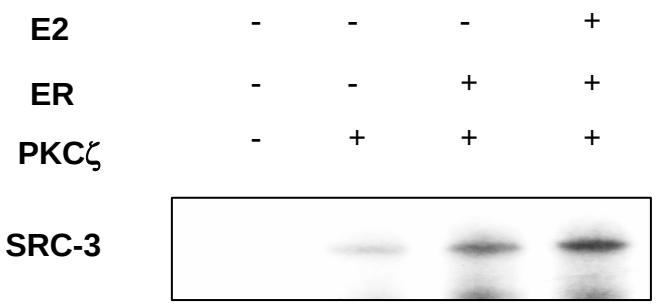


Fig. 2 (Continued)

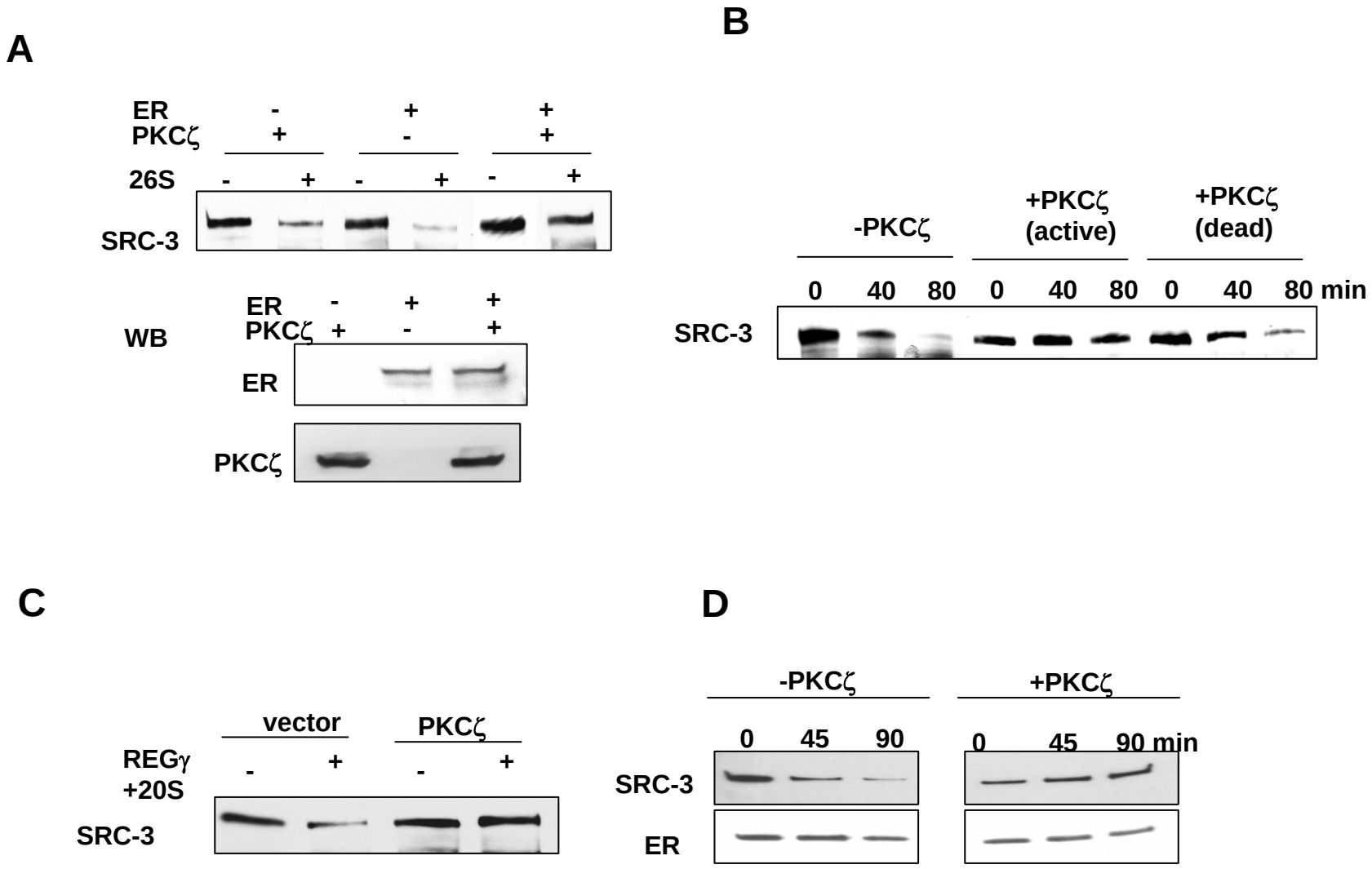
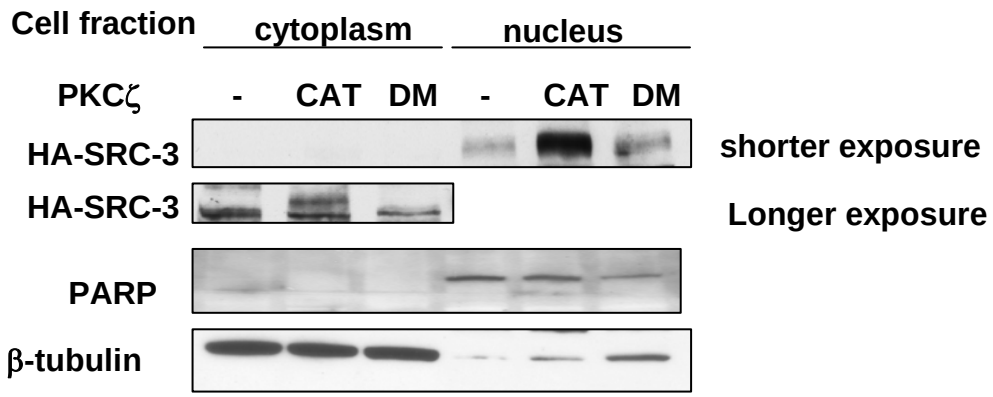

A
ER
PKCζ
26S
SRC-3
WB
ER
PKCζ
B
-PKCζ
+PKCζ (active)
+PKCζ (dead)
0 40 80 0 40 80 0 40 80 min
SRC-3
C
vector
PKCζ
REGγ +20S
SRC-3
D
-PKCζ
+PKCζ
0 45 90 0 45 90 min
SRC-3
ER

Fig. 3

A


Cell fraction
cytoplasm
nucleus
PKCζ
-
CAT
DM
-
CAT
DM
HA-SRC-3
shorter exposure
HA-SRC-3
Longer exposure
PARP
β-tubulin

B

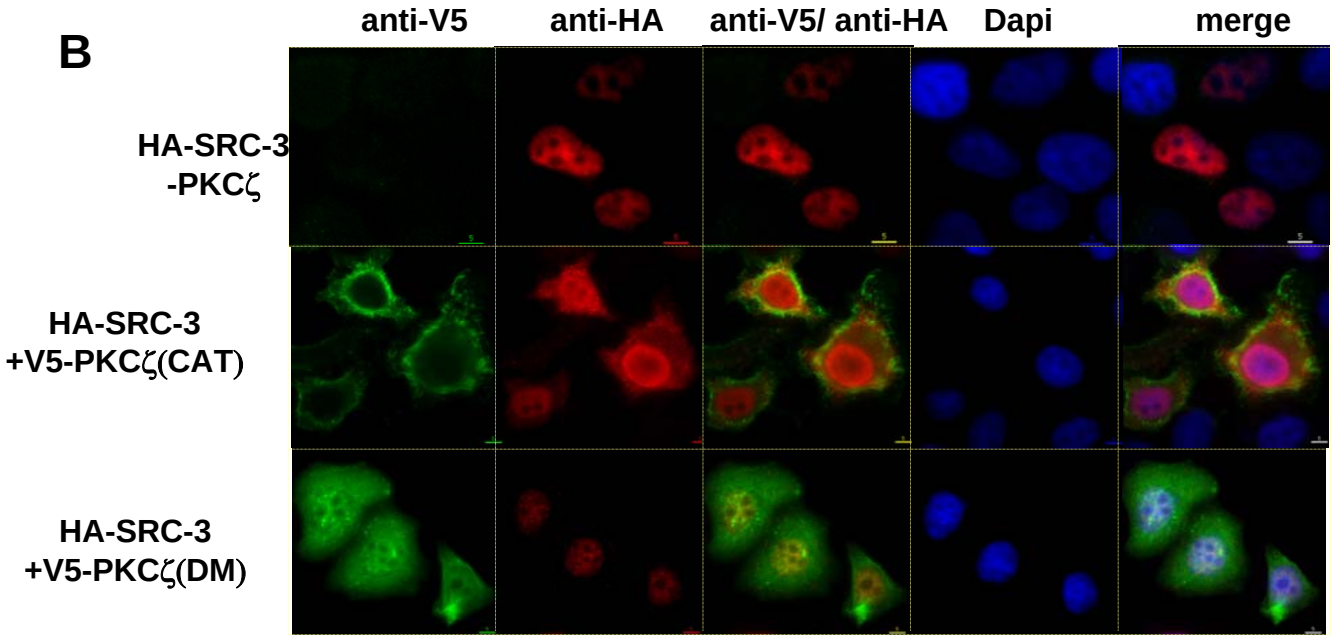

anti-V5
anti-HA
anti-V5/ anti-HA
Dapi
merge
HA-SRC-3
-PKCζ
HA-SRC-3
+V5-PKCζ(CAT)
HA-SRC-3
+V5-PKCζ(DM)

Fig. 4

C

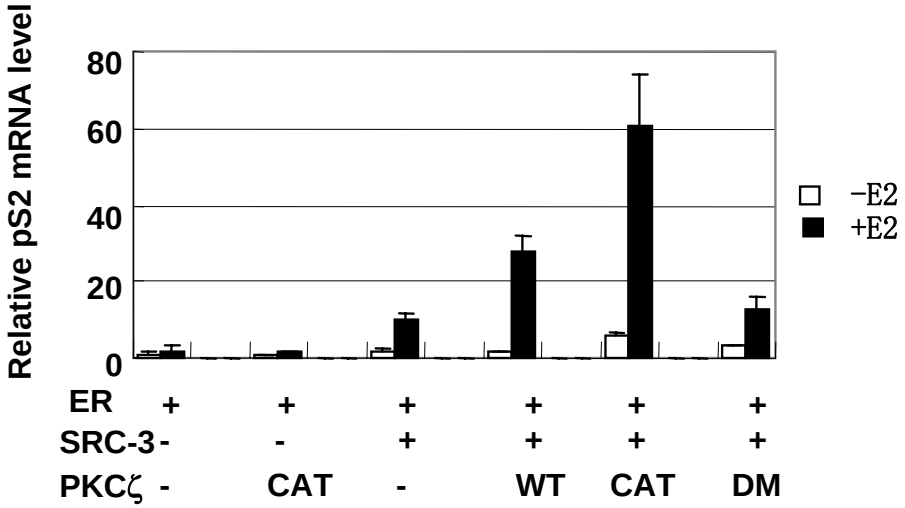


Fig. 4 (Continued)